Role of Toll like receptor 4 and interleukine-1A polymorphisms in Generalized Aggressive Periodontitis in a Romanian Population

- SUMMARY OF PHD. THESIS -

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Role of Toll like receptor 4 and interleukine-1A polymorphisms in Generalized Aggressive Periodontitis in a Romanian Population

Introduction

Periodontitis is an inflammatory oral disease that involves gingival inflammation and progressive loss of the alveolar bone around the teeth. Lack of proper treatment can lead to mobility and subsequent loss of teeth especially in the aggressive form. Generalized Aggressive Periodontitis (GAgP) involves in general young people otherwise healthy, progresses rapidly, occasionally presenting alternating acute and stable chronic phases. It appears that the prevalence of Aggressive Periodontitis among individuals younger than 35 years ranges from approximately 1% to a maximum of 15% depending on age and study (Demmer and Papapanou, 2010).

The aetiology of this disease is multifactorial. The role of bacteria is important in the pathogenesis though probably insufficient to cause periodontal disease, a susceptible host is also needed (Haffajee and Socransky, 1994). Periodontal disease is triggered by a specific host-dependent immune response that is influenced by genetic predisposition, which is evidenced by family aggregation and differences between ethnic groups.

Epidemiologic and immunogenetic approach consider the detection of an association a crucial step in understanding disease etiology, rather that a means to establish causality (Holly et al., 2002). The first step is to identify potentially relevant genes. The selection of genes was based on a careful consideration of current knowledge of disease genotype, expression studies and infectious disease models.

Polymorphism in the IL-1 gene cluster (IL-1A, IL-1B, IL-1RN) have been suggested to influence the pathogenesis of Generalized Aggressive Periodontitis (GAgP), however the results are still
conflicting. From this cytokine family we choose to study the gene polymorphism IL-1A that encodes the cytokine IL-1α. IL-1α is a pleiotropic cytokine involved in various immune responses, inflammatory processes and hematopoiesis. The pro-inflammatory and bone resorption properties of IL-1alpha strongly suggest a role for this cytokine in the pathogenesis of periodontal disease.

The most well-studied variation affecting the IL-1A gene is the change of a single nucleotide (SNP) in a region of regulatory DNA near the start of the gene, called IL1A-889 C>T (rs 1800587). This variation affects the production of IL-1α with cells. It is unclear how changes in the IL1A gene influence the risk of inflammatory disorders. Studies suggest that the effect of IL-1A variation is probably related to the role of IL-1α in promoting inflammations (U.S. National Library of Medicine, 2013).

Because Il1A (-889) is situated in the promoter region of the gene, it has been suggested that this SNP may give rise to the differential production of IL-1α by influencing gene transcription. It is a non-coding SNP (untranslated region 5’) that could alter the transcription of the IL-1A gene in the 5’ flanking region, resulting in the aberrant production of IL-1α in some diseases.

Toll-like receptors (TLRs) family recognizes a set of structurally conserved pathogen-associated molecular patterns (PAMPs) and this way play an essential role in detecting microorganisms and initiating inflammatory response (Medzhitov et al., 1997). From this family we choose to study the gene polymorphism that is encoding receptor TLR4.

This was due to its fundamental role in pathogen recognition and activation of innate immunity, mediation of cytokine production necessary for the development of the effective immunity, its implication in signal transduction events induced by LPS found in Gram negative bacteria, its association with differences in LPS responsiveness, its multiple transcripts variants and different isoforms encoding.

4
TLR4 contains two well studied SNPs at positions +896 (A>G) and +1196 (C>T), better known as TLR4 Asp299Gly (rs 4986790) and the TLR4 Thr399Ile (rs 4986791), respectively. Both SNPs are functional polymorphisms and cause amino acid changes in the extracellular domain of TLR4 and have been associated with LPS hyporesponsiveness in humans (Schwartz, 2001).

Because some of the results in the literature were conflicting, we decided to check them in a Romanian population group. So we chose to study TLR 4 genotype for Asp299Gly and Thr399Ile and IL-1A (-889) involvement in predicting susceptibility to GAgP in a Romanian ethnic group.

Methods

Subjects selection

Of the 65 Romanian subjects studied, 44 were diagnosed with GAgP and were considered to be the cases group and 21 were healthy subjects form periodontal point of view and were considered to be the controls group, according to validated clinical and radiographic criteria.

The mean age was 38,13 for the cases group and 25,61 for the control group. The gender distribution was consisting of 24 (54,55%) females and 20 (45,45%) males in the cases group, 17 (80,96%) females and 4 (19,04%) males for the controls group, respectively.

PAg affects in general the young people, healthy from systemic point of view, but it can also appear at an older age. The clinical aspects include accentuated loss of the alveolar bone and also important gingival attachment loss, in a very short time and quite severe (Armitage, 1999).

The clinical criteria was considered to be interproximal attachment loss affecting at least three permanent teeth other than a first molars and incisors. The clinical investigation included also smoking
status, plaque and bleeding indexes, pocket depth and the presence of dental mobility.

The subjects were recruited from the patients addressing regularly to a private dental clinic from the city of Iasi, Romania. The subjects that did not present loss of gingival attachment for more than one tooth, and the periodontal probe measurement was not deeper than 3mm, did not present gingival bleedings and bone resorption or a history of periodontal disease, were considered healthy from periodontal point of view and included in the control group.

Subjects presenting mental disorders, lack of judgement, minors, pregnant woman, other ethnical groups and immigrants were not selected to participate in our study. We also excluded the subjects with diabetes or the ones having antibiotic treatment in the last six months. All participants signed a written informed consent to participate in the study. The study was approved by the Medical Ethical Committee of University of Medicine and Pharmacy “Gr. T. Popa” Iasi.

Saliva samples

At this stage of knowledge saliva seems to be a highly important possible tool for regular screening of larger populations as a diagnostic and monitor disease progression tool. The most important thing is that is a simple, cheap and noninvasive method for DNA collection.

Whole saliva samples (1ml) were collected from all the subjects, both cases and controls groups, in a sterile tube and frozen until processing. The subjects were not allowed to eat or to brush 30 minutes before sampling.

DNA extraction

Genomic DNA was extracted out of the saliva samples using the kit Charge Switch gDNA Buccal Cell Kits, Invitrogen. Lysis, binding with magnetic beads, washing, eluting and quantifying DNA
were processed accordingly to the manufacturer’s instructions (User Guide- Invitrogen, Life Technologies, 2005). The DNA was quantified using the spectrophotometer NanoDrop 2000 and frozen at -20º C until the Real-Time PCR reactions were performed.

Genotyping and polymorphism analyses

The three genotypes were identified by Real-time PCR and Tagman Genotyping Assays (Invitrogen, Applied Biosystems) according to the TaqMan protocol in 96 well plates, in order to detect the A>G (Asp299Gly) and C>T (Thr399Ile) missense SNPs for TLR4 gene and C>T (-889) SNP for IL1A gene.

We took 4 µl of the each sample and we add 12,5 µl TaqMan Genotyping Master Mix, 1,25µl SNP Genotyping Assay (specific for each SNP), 11,25µl DNase-free water and the DNA samples. We obtained a PCR mix of 25 µl in each microtube. Then we used a LightCycler 480 (Roche) termocycler to analyse our 65 samples at the following parameters: 94ºC for 5 min, followed by 35 cycles :94 ºC for 30 sec, 55 ºC for 30 sec and 72 ºC for 30 sec, and a final incubation at 72 ºC by 7 min followed by a cooling to 4 ºC. A no template control (NTC) tube was used as quality control of the assay for each genotype.

Since allele B (rare) is the one hypothesized to be linked to the pathogenesis of AgP, we grouped genotypes according to the presence or the absence of allele B, in AA (wild type), AB (heterozygosity) and BB (mutant). Mx Pro software (Mx3005P, Stratagene, Agilent Technologies) was used for processing the data obtained from the DNA samples and generate the genotyping results (Fig.9.23). For better visualize the SNP associations we used also bioinformatic application Gitools for constructing a heat map of the genotype distribution.
Fig. 9.23. PCR real-time amplification plots

**Statistic Analyses**

To test for the differences in allele/genotype/canier frequencies between the cases and controls group, we calculated the adjusted Odds ratio (OR) and 95% confidence interval (CI). The incidence of polymorphisms was analyzed by Fisher’s exact test and Chi square test, and also the specificity, sensibility and relative risk (RR) were calculated. Differences were considered significant for a two sided P value equal or less then 0.05.
The statistical analyses were performed using Graphpad InStat Soft for windows software. Carrier status was considered if any subject inherited at least one copy of the variant allele B in both groups. Carrier trait analyse was performed to determine whether combinations of SNPs were acting synergistically on the risk to develop GAgP.

Results

From gender point of view, females (54, 55%) were more affected by GAgP than male (45,45%). Smoking status was present in the cases group in 20,45% and in the control group in 14,28%.

Single gene analysis

No difference was found between observed and expected distribution of genotypes, so controls were considered to be in Hardy Weinberg equilibrium and accordingly to Mendelian inheritance principles ($\lambda^2$ IL-1A (-889): 0,11; $\lambda^2$ TLR4 Asp299Gly: 1,99) , except for TLR4 Thr399Ile that could not be determined.

For the TLR4 Thr399Ile, the negative genotype was more prevalent in controls (100% vs. 10% in cases) and the positive genotype was found exclusively in the cases group (10%), which might show a trend for association with the susceptibility to GAgP but it did not reach statistic signification either (p: 0,29; OR: 5,05; 95% CI: 0,25-98,73) (table 10.4)

Table 10.4. Dominant model for TLR4 Thr399Ile polymorphism

<table>
<thead>
<tr>
<th>TLR4 Thr399Ile</th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT+TT</td>
<td>4(10%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>CC</td>
<td>36 (90%)</td>
<td>20 (100%)</td>
</tr>
</tbody>
</table>
The prevalence of the negative genotype for TLR4 Asp299Gly was higher in cases (36,58% vs. 25% in controls), and for the positive genotype the prevalence was higher for the control group (75% vs. 63,41% in cases) which might be associated with a protective effect against GAgP, but the difference was not significant from statistic point of view (p: 0,53; OR: 0,57; 95% CI: 0,15-2,11) (table 10.7).

Table 10.7. Dominant model for TLR4 Asp299Gly polymorphism

<table>
<thead>
<tr>
<th>TLR4 Asp299Gly</th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG+GG</td>
<td>26 (63,41%)</td>
<td>12 (75%)</td>
</tr>
<tr>
<td>AA</td>
<td>15(36,58%)</td>
<td>4(25%)</td>
</tr>
</tbody>
</table>

In what concerns our third SNP, IL1A (-889), negative genotype was predominant for the cases group (9,3% vs. 4,76% in controls). In what concerns positive genotype the prevalence was higher for the controls (95,23% vs. 90,69% in cases), but the difference was too low to be significant (p:1; OR: 0,48; 95% CI: 0,05-4,65).(table 10.10)

Table 10.10. Dominant model for IL1A(-889) polymorphism

<table>
<thead>
<tr>
<th>IL1A(-889)</th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT+TT</td>
<td>39 (90,69%)</td>
<td>20 (95,23%)</td>
</tr>
<tr>
<td>CC</td>
<td>4 (9,30%)</td>
<td>1 (4,76%)</td>
</tr>
</tbody>
</table>

The distribution of genotypes and Fisher exact test analyses for cases and controls is shown in table 10.12.

From all the three SNPs, TLR4 Thr399 Ile had the higher relative risk 1,55 considered to be moderate.
Table 10.12. Statistic analysis of genotypes distribution for dominant model with Fisher’s exact test

<table>
<thead>
<tr>
<th>SNP</th>
<th>P</th>
<th>OR (CI)</th>
<th>RR</th>
<th>Sensibility</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr399Ile</td>
<td>0,29</td>
<td>5,05 (0,25-98,73)</td>
<td>1,55</td>
<td>0,1</td>
<td>1</td>
</tr>
<tr>
<td>Asp299Gly</td>
<td>0,53</td>
<td>0,57 (0,15-2,11)</td>
<td>0,86</td>
<td>0,63</td>
<td>0,25</td>
</tr>
<tr>
<td>IL1A(-889)</td>
<td>1</td>
<td>0,48 (0,05-4,65)</td>
<td>0,82</td>
<td>0,90</td>
<td>0,04</td>
</tr>
</tbody>
</table>

Allelic distribution of the three studied polymorphisms

We detected the mutant allele T for TLR4 Thr399Ile only in GAgP group, the rare allele being absent in control group (5% vs. 0% in controls) . Common allele C was more prevalent in the controls group (100% vs.95% in cases). So the rare allele T showed a weak association with GAgP (p: 0,29; OR: 0,2; 95%: 0,01-3,99) (table 10.16).

Table 10.16. Allelic distribution of the SNP TLR4 Thr399Ile

<table>
<thead>
<tr>
<th>Thr399Ile</th>
<th>C</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases (80)</td>
<td>76 (95%)</td>
<td>4 (5%)</td>
</tr>
<tr>
<td>Controls (40)</td>
<td>40 (100%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

The rare allele G of TLR4 Asp299Gly was higher in controls than in patient (59% vs. 41%) suggesting a protective factor for GAgP.
The frequencies and carriage rates of common allele A were higher in GAgP patients then controls (59% vs. 41%), but when we compare the two groups the difference did not reach statistic significance (p: 0,09, OR: 0,06; 95% CI: 0,89-4,73) (table 10.17).

Table 10.17. Allelic distribution of the SNP TLR4 Asp299Gly

<table>
<thead>
<tr>
<th></th>
<th>Asp299Gly</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>Cases (82)</td>
<td>48 (59%)</td>
<td>34 (41%)</td>
<td></td>
</tr>
<tr>
<td>Controls (32)</td>
<td>13 (41%)</td>
<td>19 (59%)</td>
<td></td>
</tr>
</tbody>
</table>

The IL-1A wildtype alleles C (19,76% in cases vs. 19,04% in controls) and mutant allele T (80,23% in cases vs. 80,95% in controls) were equally distributed among cases and controls (p: 1 OR: 1,04; 95% CI: 0,41-2,66) (table 10.18).

Table 10.18. Allelic distribution of the SNP IL1A (-889)

<table>
<thead>
<tr>
<th></th>
<th>IL1A(-889)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>Cases (86)</td>
<td>17 (19,76%)</td>
<td>69 (80,23%)</td>
<td></td>
</tr>
<tr>
<td>Controls (42)</td>
<td>8 (19,04%)</td>
<td>34 (80,95%)</td>
<td></td>
</tr>
</tbody>
</table>

The distribution of allele frequency and Fisher exact test analyses for cases and controls is shown in table 10.19.
Table 10.19. Statistic analysis of allelic distribution with Fisher’s exact test

<table>
<thead>
<tr>
<th>SNP</th>
<th>P</th>
<th>OR (CI)</th>
<th>RR</th>
<th>Sensibility</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr399Ile</td>
<td>0,29</td>
<td>0,2 (0,01-3,99)</td>
<td>0,95</td>
<td>0,65</td>
<td>0</td>
</tr>
<tr>
<td>Asp299Gly</td>
<td>0,09</td>
<td>0,06 (0,89-4,73)</td>
<td>1,44</td>
<td>0,78</td>
<td>0,35</td>
</tr>
<tr>
<td>IL1A(-889)</td>
<td>1</td>
<td>1,04 (0,41-2,66)</td>
<td>1,03</td>
<td>0,68</td>
<td>0,33</td>
</tr>
</tbody>
</table>

However we found no evidence that the gene polymorphism of IL-1A and TLR4 has any significant correlation with GAgP. The sensitivity and the specificity found in both genes were low. Relative risk was also low, except for Thr399Ile who presented a moderate relative risk (1, 52).

**Multiple gene analysis**

Multiple polymorphisms in one gene could influence in an increasing manner gene expression, protein expression or protein function, so we analyzed the co-segregation genotype for TLR4 (Asp299Gly and Thr399Ile) in our study groups.

We found association for the negative genotype which was more increased in cases (34, 14% cases vs. 25% controls) and the positive genotype association was present only for the cases group (7, 31% cases vs. 0% controls). This can evidence some correlation with GAgP but not from statistical point of view.
Another association was also evidenced for the carrier genotype of TLR4 Asp299Gly and IL1A (-889). Negative genotype was present only in the cases group (2.43% cases vs. 0% controls) and the positive genotype was more frequent in the control group (75% controls vs. 56.09% cases), so it might have some protective effect.

The third composite positive genotype studied (TLR4 Thr399Ile and IL-1A (-889)) was present only in the cases group (9.75% vs. 0% in controls) which might represent a trend to susceptibility for this association and GAgP.

We also checked for the association between the 3 SNPs, this being found only for the cases group, both for negative genotype (2.43%) and positive genotype (7.31%), showing some tend into the correlation with GAgP, but not in a significant manner.

Gitools is a framework for analysis and visualization of multidimensional genomic data using interactive heat-maps (Perez-Llamas and Lopez-Bigas, 2011), so we built one for our SNPs (Fig.10.19).

No statistical difference was observed when comparing the control and GAgP groups whether or not the habit of smoking was considered.

The genetic polymorphism in these three loci may not be useful in prediction the susceptibility and severity of periodontal disease in a Romanian population.
**Fig. 10.19.** Gitools diagram of genotypes distribution for TLR4 Thr399Ile, TLR4 Asp299Gly and IL-1A (-889) in a Romanian population. Legend of genotype representation: blue AA, green AB, red BB, white PCR- Real Time non-amplificated sample, M – controls group, P – cases group.
Discussions

There is strong evidence that genetic as well as environmental factors affect the development of periodontal disease, and also some suggestion that aggressive and chronic forms of the disease share the same genetic predisposition (both share and unique genetic associations in these forms of Periodontitis).

The candidate gene approach of common SNPs can detect small to moderate relative risk in the context of aetiological and genetic heterogeneity.

Susceptible proteins are more likely to be encoded by essential genes, are more central in protein-protein interaction networks and are less likely to contain loss-of-function mutations in healthy individuals.

Proteins involved in multiply pathways are less able to tolerate mutations as more of the sequence is required for function. Proteins with fewer interactions are less constrained and so more able to tolerate mutations (Fig.11.4).

TLR4 SNPs Asp200gly and Thr399Ile are functional non-synonimous polymorphisms, their effect consisting in an amino acid substitution (D299G and T399I) which has the potential to affect protein function. Change of protein function by SNP may explain most disease-related mutations. Co-segregating missense TLR4 polymorphisms Gly-299 and Ile-399 affecting the extracellular domain of TLR4 receptor, are associated with a blunted response to inhaled LPS in humans, especially Asp299Gly interrupts TLR4 mediated LPS signaling (Arbour et al., 2000).

However heterozygous carriage of the TLR4 +896 (A> G) SNP does not affect LPS responsiveness and only the rare homozygous carriers are less responsive to LPS (Imahara et al., 2005). But the same SNP has been associated with increased susceptibility to severe Gram-negative bacterial infections (Doreen et al., 2002).
In this study we noticed a trend in the association of TLR4 Asp299Gly with a protective effect to GAgP. This might be due to the correlation of pro-inflammatory cytokine production in response to exposure to LPS, resulting in less bystander damage and in a decreased risk for GAgP (James et al., 2007). Other research also proposed the Asp299Gly polymorphism in the TLR4 gene to confer protection in other conditions associated with inflammation including acute coronary events (Ameziane et al., 2003) and progressive atherosclerosis (Arbour et al., 2000).

It should be noted that conflicting reports have been published on the role of TLR4 polymorphism in periodontal disease. A possible explanation for this can be the different types of LPS and of TLR4 expressions.
Increased production of IL-1alpha, associated with polymorphism of the IL-1 gene, has been implicated in the pathogenesis of periodontal damage (Shirodaria et al., 2000). SNPs from regulatory regions (promoter region), like IL1A (-889) can cause changes in gene expression and are essential for the regulation of the transcription of the coding region. R-allele of IL1A (-889) will result in up-regulating of protein production.

The carriage rate of R-allele of polymorphic IL1A (-889) varies from 34% to 64% for patients and 35% to 60% for controls for Caucasian subjects (Lindhe et al., 2008).

For Romanian population, R-allele of IL1A (-889) was almost equal in frequency between patients group (80,23%) and controls group (80,95%). This shows that carriage rate of genetic polymorphism may vary a lot among different ethnic populations.

Kornman et al., (1997) was the first that reported the association of the composite positive genotype of IL1A gene at nucleotide position -889 and of IL1B gene at nucleotide position +3954 with severity of periodontitis in non-smoking Caucasian patients. Meisel et al., (2002) found similar results but in smokers. Also studies have been reported a more often harbored putative periodontal pathogens and an increased counts of these pathogens in association with IL1 composite genotype (Socransky et al., 2000).

Laine et al. (2001) reported increased composite genotype of the R-alleles of the IL1A, IL1B and IL1RN genes in non-smoking patients in whom P. gingivalis and A. actinomycetemcomitans could not be detected. These results suggest that IL1 gene polymorphisms may play a role in the absence of other (putative) risk factors (Meisel et al., 2004).

However, the prevalence of genotype-positive of IL1A (-889) in different ethnic groups, and their correlation to clinical manifestations of GAgP, had displayed contradictory results.
Despite the genetic background of TLR4 gene and periodontitis, Asp299gly and Thr399Ile failed to be associated with GAgP in a Romanian group. IL1A polymorphism seemed to be equal distributed in the Romanian population between cases and controls, fact that could not permit any conclusions regarding its effect on GAgP.

These results are in concord with other studies of the same polymorphisms but of other population groups (Imamura et al., 2008, Gonzales et al., 2003).

The increased prevalence of periodontitis in young smokers was evidenced by Haber et al., (1993). Also some studies confirmed the importance of smoking as a factor in severe loss periodontal attachment in AgP (Mullally et al., 1999). In our study, smoking could not be statistically correlated with GAgP.

In polygenic diseases, such as Periodontitis, a genetic variation may be insufficient to cause disease when taken individually. However a combination of certain environmental factors and gene polymorphisms may determine the susceptibility, resistance and severity to an inflammatory process (Wang et al., 2000, 2001, 2002, 2003).

Recently published studies have shown the importance of the composite genotype (Hedley et al, 2002) and carrier trait analysis (El-Omar et al., 2003) in the search for disease susceptibility genes.

**Conclusions**

The present study failed to find any significant association between the TLR4 and IL-1A polymorphisms and GAgP, potentially because of the small sample size.

To the best of our knowledge this was the first study to examine the prevalence of these polymorphisms in a Romanian population with GAgP.
Combination of multiple polymorphisms across multiple genes may influence pathogenesis. The two and three loci could not reach statistical significance because of the too small number of samples, but we could notice some weak association of the positive genotype for the three SNPs association exclusively in the cases group. Subject carrying this composite genotype were most severely affected by GAgP. This kind of analysis should always be viewed in relation with other genes and predisposing factors.

These findings are in contrast with some other reports, this may be the result of differences between the population studied, subject criteria selection or disease characterization. Also the small sample size of many studies, including ours, and the variability of the outcomes, point out the need for large multicenter collaborative studies, adequately powered to reduce the effects sampling variation and to be able to define reliably association between TLR4 and IL-1A polymorphisms and GAgP.

The rapid advances in the field of human genetics have offered us new opportunities to investigate the role of various immune mediators in disease susceptibility and severity. Combining different studies and different research methods provides a valuable insight into the complex and dynamic host-pathogen interactions.

Modern bioinformatic tools are valuable in modeling the multifactorial and complex nature of periodontal disease. This insight might help for further understanding of Aggressive Periodontitis pathophysiology and immunogenetic, and also may help identify new research targets and define potential therapeutic targets.

**Keywords**

Generalized Aggressive Periodontitis, Toll-like receptor 4, Interleukine-1A, polymorphism
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User guide – Charge Switch gDNA Buccal Cell Kits for purification of genomic DNA from human buccal swabs, Invitrogen - Life Technologies, 2005


